Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion

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Running title: Raf-1-induced growth arrest in HMEC

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Keywords:
conversion
senescence
immortalization
Raf-1
$p16^{INK4a}$
human mammary epithelial cells

ABSTRACT

Using an estrogen-inducible retroviral system, we demonstrate that oncogenic Raf-1 induces growth arrest and morphological changes in finite lifespan human mammary epithelial cells (HMEC). This arrest does not rely on expression of the cyclin-dependent kinase inhibitor (CKI) $p16^{INK4a}$, nor on changes in expression of the CKIs $p21^{Cip1}$, $p14^{ARF}$, $p27^{Kip1}$, or $p57^{Kip2}$. The Raf-induced arrest is independent of viral oncogene mediated inactivation of p53 and pRB, or c-myc overexpression. Flow cytometric analysis demonstrates that cells arrest in both G1 and G2. The Raf-induced arrest is mitigated or eliminated in some immortally transformed HMEC. Immortal HMEC that have both overcome replicative senescence and undergone the recently described conversion process maintain growth in the presence of transduced oncogenic Raf-1; they also gain EGF-independent growth and a low frequency of anchorage-independent growth. However, HMEC that have overcome replicative senescence but have not undergone conversion, and HMEC immortalized by transduction with the catalytic subunit of telomerase, hTERT, remain severely growth arrested. These results indicate that the molecular mechanisms responsible for the Raf-1-induced growth arrest may vary among different finite lifespan cell types, and that in HMEC, this mechanism is altered during the conversion process, rather than as a direct consequence of overcoming senescence or expressing hTERT.

INTRODUCTION

Overexpression of activated forms of Ras and Raf induces malignant progression in a variety of immortally transformed rodent and human cell lines (Khosravi-Far et al., 1996; Samuels et al., 1993). Conversely, transduction of activated Ras or Raf into finite lifespan human or rodent cells, as well as some immortal human cell lines, can induce cessation of growth associated with a phenotype resembling senescence (Lin et al., 1998; McMahon & Woods, 2000; Ravi et al., 1999; Zhu et al., 1998). This differential effect suggests that significant alterations may occur in cellular responses to overexpressed Ras and Raf when finite lifespan cells transform to immortality. Such alterations could have major consequences for tumorigenic progression. In vivo, the growth inhibitory effect of activated Ras and Raf in finite lifespan cells may be due to a tumor suppressor mechanism that halts proliferation in the presence of abnormal signaling. Alteration of this mechanism may allow deregulated growth.

Studies on the downstream effectors of activated Raf have indicated the involvement of the MEK/MAPK signaling pathway for both growth arrest and the induction of malignant progression in different cell types (Kolch et al., 1991; Zhu et al., 1998). Initial reports on Ras/Raf-induced growth arrest of finite lifespan human fibroblasts and primary rodent fibroblasts suggested that this "premature senescence" proceeded through induction of the CKIs $p16^{INK4a}$ and/or $p21^{Cip1}$ (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998). This suggestion was based on the observations that Ras/Raf-induced arrest was accompanied by elevation of p16 and p21 levels, and that overexpression of p16 alone was sufficient to induce a senescence-like growth arrest in normal human lung fibroblasts (Zhu et al., 1998). More recent research has shown that in human astrocytes in which the p16/pRB pathway has been compromised, Raf can still induce growth arrest, accompanied by p53-independent induction of p21 (Fanton et al., 2001).

It is currently not known what changes occur in immortal cells to alter the normal growth inhibitory response of finite lifespan cells to oncogenic Ras/Raf and to permit continued proliferation and malignant progression. Primary rodent fibroblasts lacking either p53 or $p16/p19^{ARF}$ are immortally and tumorigenically transformed in response to oncogenic Ras or MEK (Lin et al., 1998; Serrano et al., 1997). Normal human fibroblasts, however, continue to exhibit growth arrest in response to oncogenic Ras when either the p53 or p16/pRB pathway is disrupted (Serrano et al., 1997).

We have used the human mammary epithelial cell (HMEC) culture system developed in our laboratory (Hammond et al., 1984; Stampfer, 1985) to determine the effect of activated Raf-1 on finite lifespan and immortally transformed HMEC. In this system, HMEC undergo a senescence-like growth arrest after ~15-25 population doublings, associated with elevated levels of p16, expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995), and a mean terminal restriction fragment (TRF) length of ~6-8 kb, but without increased expression of p21 or p53 (Romanov et al., 2001). When grown in a serum-free medium (Hammond et al., 1984), some HMEC can undergo a spontaneous selection for down-regulation of p16 expression, associated with methylation of the p16 promoter (Brenner et al., 1998). The resulting post-selection cells continue to grow for ~30-70 additional population doublings, depending upon the individual donor strain. Post-selection HMEC populations experience a second proliferation barrier when their telomeres become critically short, mean TRF $\leq$5 kb (Romanov et al., 2001; Stampfer et al., 1997). This second senescence block, termed agonescence (Tlsty et al., 2001) is again associated with expression of SA-β-gal, but is not associated with de-repression of p16. Agonescence is extremely stringent; we have never observed spontaneous escape from agonescence in untreated HMEC cultures from normal tissue.

In very rare instances, cells have escaped agonescence in HMEC cultures treated with chemical carcinogens. However, our recent studies have indicated that even after overcoming both senescence barriers, the resultant HMEC with indefinite proliferative potential, if p53(+), must proceed through a very gradual process, which we have termed conversion, in order to reactivate telomerase and attain uniform good growth (Garbe et al., 1999; Nijjar et al., 1999; Stampfer & Yaswen, 2000; Stampfer et al., 1997). Conversion appears to be triggered by the

critically short telomeres (mean TRF < 3 kb) that p53(+) HMEC that have recently overcome agonescence incur by maintaining proliferation in the absence of telomerase activity. We have called these telomerase(-), p53(+) HMEC populations conditionally immortal, since not all individual post-agonescence cells maintain indefinite proliferation. In immortal HMEC that are p53(-/-), the conversion process is greatly accelerated, and some aspects are not expressed. As a result, uniformly good-growing, telomerase-expressing cells appear much more rapidly (Stampfer et al, submitted for publication).

We have also immortalized post-selection p16(-) HMEC by transduction of hTERT, the catalytic subunit of the telomerase complex. This method of immortalization bypasses the agonescence block and the conversion process in post-selection HMEC (Stampfer et al., 2001). Transduction of pre-conversion populations of conditionally immortal HMEC line 184A1 with hTERT also bypasses the conversion process; however, introduction of hTERT into 184A1 once conversion has begun does not prevent the conversion process from proceeding (Stampfer et al., 2001).

In the current study, we sought to determine whether finite lifespan p16(-) HMEC would still exhibit growth arrest when exposed to activated Raf-1, and if immortally transformed HMEC could escape Raf-induced growth arrest. We found that post-selection p16(-) HMEC were growth-arrested in both G1 and G2, and that neither RB nor p53 function were required for this arrest. Moreover, pre-selection HMEC, still capable of expressing p16, were also growth-inhibited in the presence of activated Raf-1 *without* exhibiting increased p16 levels. We also determined that fully immortal, telomerase(+) HMEC lines that had undergone the conversion process could maintain growth in the presence of oncogenic Raf-1, and displayed evidence of further malignant progression. However, early passage, telomerase(-), conditionally immortal populations that had not yet undergone the conversion process were growth-inhibited similar to the finite lifespan cells. hTERT-immortalized cells that had bypassed conversion were also growth-inhibited. Thus the alteration in cellular physiology that allowed HMEC to maintain growth in the presence of transduced oncogenic Raf-1 occurred during the process of conversion, and not as a consequence of overcoming agonescence or expressing telomerase activity.

RESULTS

Oncogenic Raf-1 induces morphological changes and suppresses growth in p16(-) post-selection HMEC.

To assay the effect of oncogenic Raf-1 on p16(-) finite-lifespan HMEC, we transduced post-selection HMEC strains 48R and 184 with a retroviral expression vector encoding a chimera consisting of EGFP fused to the catalytic domain of human Raf-1, in turn fused to the hormone-binding domain of the human estrogen receptor (hereafter referred to as Raf:ER; (Samuels et al., 1993)). The Raf-1 fragment used in these experiments had two adjacent tyrosine residues (Y340 and Y341 [YY] in the full-length Raf-1) mutated to aspartic acid [DD], causing increased Raf-1 activity (Woods et al., 1997). The Raf:ER protein possesses little or no kinase activity until induced with estrogen or its analog 4-hydroxy-tamoxifen (4-HT). Control cells were infected with a retroviral vector encoding only the hormone-binding domain of the human estrogen receptor (hbER). As an additional control, Raf:ER cells were treated with ethanol vehicle alone.

Induction of Raf[DD]:ER-transduced 48R with 100 or 500 nM 4-HT produced dramatic morphological alterations; cells became enlarged and often elongated, with a flattened, vacuolated morphology (Figure 1D). Cells treated with ethanol or transduced with the hbER control retroviral vector showed normal morphology and growth patterns (Figure 1A-C). Compared to controls, cell numbers were dramatically decreased in Raf:ER-transduced 48R treated with 100-500 nM 4-HT for 6 days (Figure 1E). At 100 nM 4-HT, there was virtually no net increase in cell numbers, and the cells remaining after 6 days of exposure to oncogenic Raf showed almost no ability to form colonies even after removal of the 4-HT (Figure 1E). Similar results were seen in Raf:ER-transduced 184 HMEC (data not shown). Indicators of metabolic function (MTT) and membrane integrity (trypan blue) indicated that nearly all the Raf:ER-transduced 48R cells remained viable, while TUNEL assays

revealed no evidence for apoptosis after 3 or 6 days of Raf induction (data not shown). Since exposure of control hbER cells to 500 nM 4-HT resulted in some growth inhibition, 100 nM 4-HT was chosen as the standard concentration for induction of Raf:ER for further experiments.

Expression of SA-β-gal has been correlated with Raf-induced growth arrest in human fibroblasts and astrocytes (Fanton et al., 2001; Lin et al., 1998; Zhu et al., 1998). However, post-selection 48RS-Raf:ER did not express increased levels of SA β-gal upon induction of Raf:ER (data not shown). This result is similar to that seen in high-grade glioma cells, which arrest in response to activated Raf but do not increase SA β-gal expression (Fanton et al., 2001).

Although the cell counts indicated severe growth inhibition induced by oncogenic Raf, when cells were induced with 4-HT for 6 days and labeled with [^{3}H]-thymidine for the final 24 hours of induction, the labeling index of Raf-exposed cells was around 50% of control values (Figure 2A). This relatively high percentage of labeled cells suggested that the growth arrest was not limited to G1. Flow cytometric analysis of control and Raf-arrested cells confirmed that after 6 or 12 days of induction of the Raf:ER fusion protein, cells accumulated in G1, S, and G2/M (Figure 2B) with a profile similar to that exhibited by normal human fibroblasts in similar studies (Zhu et al., 1998).

p16 is not expressed in Raf-arrested post-selection HMEC.

Studies on Ras- and Raf-induced arrest in various rodent and human cell types suggested that this arrest might be mediated by one or more CKIs, including p16, p21, p15, and p27 (reviewed in (McMahon & Woods, 2000)). Normal human fibroblasts expressed elevated levels of p16 upon induction of Raf:ER; increased p16 levels are also observed at senescence of some fibroblasts (Zhu et al., 1998). Because post-selection HMEC do not express p16, even at agonescence, we assayed whether activated Raf could induce p16 in these cells. Western blot analysis revealed no p16 expression in Raf-arrested post-selection 48R-Raf:ER (Figure 3); therefore the growth arrest must be mediated by other factors. One possibility was p21, since p21 expression is increased in normal human astrocytes upon Raf induction and may contribute to Raf-induced growth arrest (Fanton et al., 2001). However, we found no change in the levels of p21 protein expression in cells arrested by activated Raf. In fact, levels of p53 protein – often implicated in p21 induction- were decreased rather than increased in the Raf-arrested cells. Levels of expression of other CKIs, p27, p57, and $p14^{ARF}$, were also examined and found not to change. The mRNA levels of p15, another CKI implicated in senescence and Ras-induced arrest (Malumbres et al., 2000) were also found to be relatively unchanged. These results indicate that Raf-induced arrest of finite lifespan human epithelial cells does not require increased expression of the CKIs whose increased expression is associated with fibroblast senescence. mRNA levels of cyclin D1, which plays a facilitating role in cell cycle progression, but which also increases during senescence (Dulic et al., 1993), were found to increase in Raf-arrested HMEC.

The MEK/MAPK kinase cascade has been implicated in Raf-induced growth arrest of various cell types through use of the MEK inhibitors PD98059 and U0126. We found that MEK is phosphorylated in Raf-arrested post-selection 48R HMEC, but we were unable to demonstrate an abrogation of Raf-induced arrest using U0126 because concentrations of this compound that may have been sufficient to prevent Raf-induced arrest profoundly inhibited the growth of these cells (data not shown).

Oncogenic Raf-1 induces morphological changes and suppresses growth in p16(+) pre-selection HMEC without increasing p16 levels, whereas Raf-arrested 48R fibroblasts upregulate p16.

Pre-selection HMEC display a gradual increase in p16 expression as they approach a senescence-like growth arrest. Since p16(-) post-selection HMEC were growth arrested by oncogenic Raf in the absence of p16 expression, we investigated whether the pre-selection HMEC, which can express p16, would induce p16 in

response to oncogenic Raf. Pre-selection cultures of 48R HMEC were infected with Raf:ER and hbER control retroviral vectors at passage 2 and induced at passage 3 with 100 nM 4-HT for 6 days.

As expected, pre-selection 48R HMEC showed complete growth arrest when Raf:ER was induced. Raf-arrested cells lost their cobblestone appearance and became elongated, forming clumps of cells with long, thin processes (Figure 4A,C). Trypan blue exclusion assays revealed these cells to be viable (data not shown). Surprisingly, their p16 protein levels decreased relative to uninduced control cells, while p21 protein levels remained constant and p53 protein decreased (Figure 4D). The percentage of SA-β-gal-expressing cells in Raf-arrested populations was difficult to determine because the clumping together of cells prevented accurate scoring of individual cells. All cellular masses examined in Raf-arrested cultures appeared to express SA-β-gal, while uninduced control cells exhibited a combination of proliferating, SA-β-gal-negative, as well as senescent, SA-β-gal-positive cells (data not shown).

Because we observed no induction of p16 in pre-selection 48R HMEC, and because p16 upregulation is observed in finite lifespan human fibroblasts arrested by Raf, we examined the response of 48R human mammary fibroblasts (48R Fb) to Raf:ER. 48R Fb were infected with Raf:ER and hbER control retroviral vectors at passage 6 and induced at passage 8 with 100 nM 4-HT for 6 days. As expected, upon exposure to 100 nM 4-HT, 48R Fb rapidly growth-arrested and became rounded, with long, thin processes (Figure 4B). Raf-arrested cells showed increased SA-β-gal expression (Figure 4C) and were viable, as determined by trypan blue exclusion (data not shown). Western blots revealed that p16 protein was induced in Raf-arrested 48R Fb, while p53 levels decreased and p21 levels showed a moderate increase (Figure 4D).

These data indicate that even though 48R pre-selection HMEC are capable of expressing p16, they do not upregulate it upon induction of Raf:ER and therefore possess a p16-independent mechanism for Raf-induced growth arrest.

Raf-induced growth arrest in HMEC is independent of viral oncogene mediated** inactivation of **p53 and pRB function, and insensitive to altered levels of c-myc.

C-myc, as well as viral oncogenes known to bind and inactivate p53 and pRB, have been shown to cooperate with Ras and Raf to transform primary rodent fibroblasts, apparently by extending the proliferative lifespan of the cells (Land et al., 1986; Serrano et al., 1997). These experiments, and others performed with primary mouse embryo fibroblasts containing targeted deletions, have led to the general hypothesis that Ras- or Raf-induced growth arrest is dependent on the function of p53 and the p16/pRB pathway (Lin et al., 1998; Serrano et al., 1997). However, in human fibroblasts, ablation of p53 and p21 by viral oncogenes fails to abrogate Raf-induced arrest (Zhu et al., 1998). To determine whether HMEC rely upon p53 or pRB for Raf-induced arrest, we transduced Raf:ER-expressing post-selection 48R with retroviruses encoding viral oncogenes known to inhibit p53 and/or pRB function: HPV16-E6 and –E7, adenovirus E1A, and SV40 large T antigen. Expression of these oncogenes, as well as disruption of p53 and pRB expression and/or function was verified by immunological and/or functional assays. HPV16-E6 and E1A containing cells did not exhibit p53 detectable by immunoblotting, nor did they undergo p53-dependent arrest following colcemid exposure (data not shown). HPV16-E7 containing cells exhibited greatly reduced levels of RB (data not shown). None of these viral oncogenes was able to prevent Raf:ER-induced growth arrest (Figure 5). Furthermore, overexpression of c-myc via retrovirus-mediated transduction was unable to rescue post-selection 48R from arrest.

Growth inhibition by activated Raf is abrogated as conditionally immortal HMEC convert to full immortality.

We utilized the immortally transformed HMEC line, 184A1, to determine whether indefinite lifespan HMEC had altered responses to oncogenic Raf, and if so, at what point in the process of attaining full immortality such changes occurred. Conditionally immortal 184A1 populations (mean TRF > 3 kb, passages 9-15) show uniform

good growth prior to the onset of conversion, although no telomerase activity is detected. The conversion process starts at approximately passage 16, when mean TRF values fall below 3 kb, and is associated with high levels of p57 expression and poor heterogeneous growth (Nijjar et al., 1999; Stampfer et al., 1997). 184A1 reproducibly converts to full immortality (uniform good growth and telomerase activity) by around passages 40-45. Conditionally immortal 184A1 at passage 12, and fully immortal 184A1 at passage 69 were infected with the hbER or Raf:ER retroviral vectors and then assayed for growth ± 100 nM 4-HT. After 6 days of induction, conditionally immortal Raf:ER-transduced 184A1 evidenced severe growth suppression, lack of colony forming ability, and a morphology similar to Raf:ER-arrested post-selection 48R and 184 HMEC (Figure 6A). In contrast, fully immortal Raf:ER-transduced 184A1 maintained some growth and colony forming ability in the presence of 100 nM 4-HT (Figure 6B). The morphology of this induced population appeared similar to the uninduced controls, although the cell numbers were reduced. Similar studies were performed in two additional immortally transformed HMEC lines, p53(+) 184B5 and p53(-/-) 184AA2. When 184B5 was first detected and assayed, it had a mean TRF of ~3 kb and was already in the early stages of conversion, exhibiting heterogeneous growth (Stampfer et al., 1997). 184B5 transduced at passage 12 showed a heterogeneous response when Raf was activated. The morphology of the activated Raf:ER 184B5 population at passage 16 illustrates this heterogeneity; some cells express a flattened, elongated appearance while others express the more normal cobblestone morphology (Figure 6C). Some cells were able to maintain growth in the presence of activated Raf, although overall growth was significantly inhibited. In contrast, fully immortal 184B5 transduced with Raf:ER at passage 46 showed some growth inhibition but no reduction in colony forming efficiency when Raf was induced (Figure 6D). 184AA2, which lacks p53 and undergoes accelerated conversion (Stampfer et al., submitted), was infected with the Raf:ER and hbER vectors at passage 54 and assayed for growth ± 4-HT. Similar to the fully immortal p53(+) lines, 184AA2 maintained growth after induction of activated Raf (Figure 6E). Altogether, these experiments indicate that the alteration(s) which allowed immortally transformed HMEC lines to gain resistance to Raf-induced growth inhibition occurred after conversion to full immortality, rather than as a consequence of overcoming agonescence.

The above experiments clearly demonstrated that fully immortal HMEC displayed altered responses when transduced with oncogenic Raf. To determine whether these altered responses might be due to differences in Raf activity in the different cell types, we examined the expression of Raf:ER and the phosphorylation status of the Raf downstream target MEK in finite lifespan, conditionally immortal, and fully immortal HMEC (Figure 7). Although all the cells were derived by infection with the same retrovirus and selection with the same puromycin concentration, basal and induced levels of Raf:ER were reduced in fully immortal 184A1 compared to finite lifespan HMEC and conditionally immortal 184A1. In all cells, MEK phosphorylation was increased within one hour of 4-HT addition and was sustained for at least 48 hours, although the levels in fully immortal 184A1 were again lower than finite lifespan or conditionally immortal HMEC. To determine whether the continued growth and survival of a proportion of the fully immortal HMEC might be due to the lower Raf:ER expression in some cells, we used the EGFP tag on the Raf:ER protein to purify by FACS a 184A1-Raf:ER subpopulation at passage 74 that expressed the highest levels of Raf:ER. When compared to the unsorted population, this purified population exhibited a lower percentage (10%) of surviving cells after 6 days of induction with 4-HT, but a higher colony-forming efficiency (100%) in the surviving cells.

hTERT-immortalized HMEC remain growth-inhibited by oncogenic Raf.

One of the hallmarks of conversion is the de-repression of endogenous telomerase activity. To determine whether acquisition of telomerase activity itself could confer resistance to growth arrest by activated Raf, we examined HMEC transduced with hTERT. Post-selection finite lifespan 184 HMEC were transduced with hTERT at passage 11 and subsequently displayed an indefinite lifespan (Stampfer et al., 2001). 184-hTERT was infected at passage 25 with the Raf:ER or hbER retroviral vectors and examined for growth and morphology. Cells expressing Raf:ER that were induced with 100 nM 4-HT showed growth arrest, very low colony forming efficiency, and a morphology comparable to that observed in Raf-induced 48R- and 184-Raf:ER (Figure 8A). These results demonstrate that hTERT-induced immortalization, which bypasses

agonescence and conversion, does not confer resistance to growth arrest in the presence of the inappropriate Raf:ER activity.

To determine whether another aspect of conversion was necessary for acquisition of resistance to Raf-induced growth arrest, we examined conditionally immortal 184A1 transduced with hTERT prior to and during conversion. Conditionally immortal pre-conversion 184A1 transduced with hTERT at passage 12 (184A1(12p)-hTERT) are able to bypass conversion and the associated period of slow heterogeneous growth (Stampfer et al., 2001). However, hTERT transduction into 184A1 at passage 22 (184A1(22p)-hTERT), when conversion has already begun, does not affect growth, and the population gradually proceeds through conversion.

184A1(12p)-hTERT cells were infected with the Raf:ER retroviral vector at passage 26, and assayed for growth ± 100 nM 4-HT at passage 35. This population, which had bypassed conversion, displayed morphological changes after Raf induction similar to those observed in finite lifespan and hTERT-immortalized HMEC (Figure 8B). However, the suppression of growth was not as severe. In contrast, when 184A1(22p)-hTERT infected with Raf:ER at passage 55 was assayed at passage 57 for growth and morphology after 6 days of induction with 4-HT, these cells, which had undergone conversion, were able to maintain growth and displayed little change in morphology (Figure 8C).

Altogether, these data support the hypothesis that obtaining the ability to maintain growth in the presence of overexpressed oncogenic Raf results from changes that occur during conversion, rather than from achieving an indefinite lifespan simply through the acquisition of telomerase activity. The data also show that the presence of transduced hTERT did not prevent the conversion-associated alteration in response to activated Raf.

Oncogenic Raf allows fully immortal 184A1 to gain some malignancy-associated properties.

To determine whether activated Raf was capable of affecting malignancy-associated pathways in immortal HMEC, assays for growth factor and anchorage independence were performed. Finite lifespan HMEC are completely dependent upon EGF receptor signal transduction for growth, as are our fully immortal HMEC lines (Stampfer et al., 1993). EGF-independent growth is an additional level of derangement exhibited by many cancer cells. Fully immortal 184A1-Raf:ER was grown in medium containing 100 nM 4-HT for one passage to select for a uniform population of cells capable of proliferation in the presence of activated Raf, while control 184A1-Raf:ER was maintained in medium with ethanol. 4-HT-grown and ethanol-treated control cells were then placed in medium minus EGF with the anti-EGF-receptor antibody MAb 225 present to block autocrine stimulation. The uninduced cells failed to proliferate, while the 4-HT-selected cells exhibited some growth in the absence of EGF receptor signal transduction (Figure 9). EGF-independent growth with induced Raf:ER was also observed in the HMEC line 184B5 (data not shown).

Anchorage-independent growth is another hallmark of neoplastic transformation. Fully immortal 184A1 show no ability to form colonies when suspended in methylcellulose. Adherent cultures of 184A1-Raf:ER at passage 75 were induced with 100 nM 4-HT for 6 days, then trypsinized and suspended in methylcellulose in the continued presence of 4-HT. After three weeks, suspension cultures were examined for the presence of colonies. A low level of colony formation was detected in the induced population, whereas uninduced controls formed no colonies (Table 1).

These results indicate that the activities of activated Raf that contribute to malignant progression were retained in fully immortal HMEC that were not growth-inhibited.

DISCUSSION

Overexpression of activated Ras or Raf may promote malignant progression of immortally transformed cells, while triggering growth arrest in finite lifespan cells. The CKIs p16 and p21 have been reported to be involved in this arrest. Our results demonstrate that post-selection HMEC, which lack p16 expression, can still be growth-arrested by activated Raf. Growth arrest is accompanied by a decrease in p53 levels, p16 expression is not regained, and the levels of p21, p27, p57, and $p14^{ARF}$ are unchanged. Arrest is independent of viral oncogene mediated inactivation of p53 and RB, and unaffected by overexpressed c-myc. Pre-selection HMEC, which are capable of p16 expression, are also growth-arrested by activated Raf without showing increased levels of p16 or p21 expression. Importantly, fully immortal HMEC lines that have undergone the conversion process can escape Raf-induced growth arrest, whereas cells from these same immortal lines, prior to undergoing conversion, remain susceptible to growth arrest.

Previous studies in other cell types showing that Raf-induced growth arrest is commonly associated with increased expression of p16 and/or p21 (Lin et al., 1998; Lloyd et al., 1997; Roper et al., 2001; Zhu et al., 1998), have led to the hypothesis that these CKIs could mediate this arrest. Human astrocytes have recently been shown to possess at least two distinct Raf-induced growth arrest pathways, one associated with induction of p16 and the other involving the p53-independent induction of p21 (Fanton et al., 2001). The p16-associated arrest that occurs in normal human fibroblasts and astrocytes with an intact p16/pRB pathway resembles senescence in that SA-β-gal is expressed and the arrest is irreversible (Fanton et al., 2001; Zhu et al., 1998). In contrast, the p21-associated arrest demonstrated in glioma cells bearing homozygous deletions of p16 is reversible and does not involve SA-β-gal expression (Fanton et al., 2001). Murine keratinocytes have been shown to depend upon both p53 and p21 for Raf-induced growth arrest, but not for Raf-induced induction of differentiation markers (Roper et al., 2001).

Our results present the possibility that additional mechanisms of Raf-induced growth arrest exist, since we show no alteration of p21 expression in Raf-arrested HMEC, and no requirement for p16 expression. Indeed, post-selection HMEC arrest in the absence of any p16 expression, and pre-selection HMEC express reduced levels of p16 upon Raf induction. In both HMEC types, the growth arrest was irreversible. Increased expression of SA-β-gal was not observed in post-selection Raf-arrested HMEC. In contrast, Raf-arrested fibroblasts obtained from the same breast tissue showed elevated p16, p21, and SA-β-gal expression. Collectively, these data indicate that different cell types may utilize different pathways to achieve growth arrest in the presence of a potentially oncogenic signal.

Diverse cell types have been shown capable of initiating different responses based on both the intensity and duration of Ras/Raf signaling (Sewing et al., 1997; Woods et al., 1997; Zhu et al., 1998). Given its pivotal role in multiple aspects of cellular physiology, it is not surprising that cells would develop mechanisms to sense imbalances in Ras/Raf signaling, and to restrain cell responses in the face of such imbalances. It is possible that the growth arrest elicited in normal cells by over-expressed or oncogenic Raf may serve not only as a tumor suppressor mechanism in cancer-prone vertebrates, but also as a way of transiently integrating internal and external stimuli. The multiplicity of mechanisms by which cells achieve growth arrest might then reflect constraints that have evolved independently in different cell types.

The fact that the Raf-induced growth arrest in HMEC occurs in the presence of viral oncogenes that inactivate p53 and/or pRB suggests that the arrest does not require intact p53 or pRB function, although the formal possibility remains that abrogation of p53 and RB functions by the viral oncogenes is not complete. These findings suggest that an uncharacterized separate tumor suppressor pathway exists and is capable of operating independently of these genes. These results differ from what has been seen in murine cells, including epithelial cell types, suggesting caution in using these rodent systems to model the human cellular processes. This is particularly important because the finding that this pathway is inducible even in instances when p53 and pRB are partially or totally disabled has obvious therapeutic implications.

A major finding of the present research is that the resistance to Raf-induced growth inhibition seen in immortalized HMEC is a consequence of events that occur during the conversion process, rather than an obligate effect of overcoming senescence or expressing telomerase activity. This is evidenced by the fact that the immortally transformed HMEC line 184A1 was severely growth-arrested in the presence of activated Raf at early passage levels prior to the onset of conversion, i.e., when the population's mean TRF was still > 3 kb and it displayed no telomerase activity. Activation of Raf in higher-passage, fully immortal 184A1 populations did not cause growth arrest, although growth was initially slower. Instead, in the latter population, activated Raf conferred the malignancy-associated properties of EGF-independent growth and a low level of anchorage-independent growth. Similarly, early passage 184B5 populations, already in the process of conversion, showed a heterogeneous response to activated Raf, whereas fully immortal 184B5 was resistant to arrest and able to grow without EGF in the presence of activated Raf. The possibility that telomerase activity by itself might affect HMEC response to Raf was ruled out by demonstrating that post-selection HMEC immortalized by hTERT transduction, and early passage pre-conversion 184A1 transduced with hTERT - immortal populations which both bypassed the conversion process - retained susceptibility to Raf-induced growth arrest. In contrast, 184A1 transduced with hTERT during conversion - and subsequently unable to bypass conversion (Stampfer et al., 2001) - displayed resistance to Raf-induced growth arrest. Collectively, these data indicate that the conversion process is necessary for immortalized HMEC to gain the ability to grow in the presence of tranduced oncogenic Raf. Further understanding of the events that occur during conversion may provide clues as to the changes that enable HMEC to proliferate in the presence of the transduced oncogenic Raf.

To our knowledge, this is the first study in which Raf:ER levels and activity have been directly compared in closely related cell types that do or do not display a growth arrest response. The means by which fully immortal HMEC escape Raf:ER-induced growth arrest may be due, in part, to reduced levels of Raf:ER expression in these cells. The lower level of Raf:ER expression in fully immortal 184A1-Raf:ER suggests that these cells, unlike the post-selection and early passage conditionally immortal HMEC, may have a mechanism for downregulating Raf:ER levels and thereby maintaining growth. Studies in NIH3T3 cells have shown that response to activated Raf can be dosage sensitive. In 3T3 cells, the dosage effect was correlated with ability to induce p21 (Sewing et al., 1997), although in HMEC, this is clearly not the case. The reason for reduced levels of Raf:ER in fully immortal HMEC is presently unknown. It is possible that available factors necessary for activation of the retroviral promoter driving Raf:ER expression or for stabilization of the fusion protein are reduced during conversion. Alternatively, repression/destabilization factors may be increased during conversion. Like all promoters, the Moloney murine leukemia virus long terminal repeat used to drive RAF:ER expression contains binding sites for cellular activators and repressors whose expression can vary with differentiated and perhaps transformed state. In future studies, it will be necessary to directly compare the levels of Raf activity attained in cells that are or are not growth arrested. In preliminary experiments, we have observed that Raf:ER-transduced post-selection HMEC treated with very low concentrations of 4-HT are able to maintain some growth, both in the presence and absence of EGF . The low-level Raf activation in this case may be sufficient to compensate for the loss of the EGF growth signal, without triggering the signal for growth arrest.

The acquisition of malignancy-associated properties in fully immortal HMEC lines expressing activated Raf is consistent with many previous studies in a wide variety of immortal cell types that have indicated that mutated or overexpressed Raf is capable of promoting malignant transformation. Activation of Raf is sufficient to promote cell cycle progression and transformation in some established cell lines (Samuels et al., 1993). Raf activation in MCF-10A cells, an immortally transformed human mammary epithelial cell line, protects these cells from detachment-induced apoptosis (anoikis), thus conferring a more transformed, rather than growth-inhibited phenotype (Schulze et al., 2001). In addition, constitutive Raf activity in MCF-7 breast cancer cells enables them to grow in the absence of estrogen (El-Ashry et al., 1997). These collective data indicate that many immortally and malignantly transformed cells have undergone a significant alteration in their response to overexpressed Raf as a consequence of transformation from the normal finite lifespan state. Consequently, examination of the Raf signaling pathways in such immortal and malignantly transformed cells can not be

assumed to present an accurate or complete depiction of these pathways in normal human cells. Gaining information on the normal cellular Ras/Raf pathways will require examination of finite lifespan cells. Such information can then form the basis for comparison in defining malignancy-associated derangements.

In conclusion, we have described the existence of an alternative mechanism of growth arrest in finite lifespan HMEC that does not require intact p53 or pRB function, p16 expression, or an increase in the levels of p21. We have also shown that HMEC acquire resistance to Raf-induced growth arrest subsequent to immortalization, during a process termed conversion, and we have ruled out telomerase activation as the component of conversion responsible for alleviating Raf-induced growth arrest. Finally, we have demonstrated that the growth effects of oncogenic Raf are dramatically different in finite lifespan HMEC compared to their immortalized, converted counterparts. The importance of using human finite lifespan cells to understand the normal Ras-Raf signaling pathways in human cells cannot be overemphasized.

MATERIALS AND METHODS

Cell culture

Finite-lifespan 184 and 48R HMEC were obtained from reduction mammoplasty tissue and grown in serum-free MCDB 170 medium (MEGM; Clonetics Division of BioWhittaker, Walkersville, MD) or serum-containing MM medium as described previously (Stampfer, 1985). The p16(-) post-selection 184 and 48R HMEC cease growth around passages 22 and 27 respectively, equivalent to about 60-80 total population doublings. The pre-selection 48R HMEC cease active growth around passages 4-5, associated with high levels of p16 expression. Fibroblasts from specimen 48 were grown in serum-containing DME/F12 medium; they cease growth around passage 20, equivalent to about 40-50 population doublings. Indefinite lifespan p53(+) 184A1, 184B5, and p53(-/-) 184AA2 cells arose independently from 184 HMEC following benzo(a)pyrene exposure of primary cultures as described (Stampfer & Bartley, 1985), and insertional mutagenesis into the p53 gene locus (Stampfer et al., submitted). These cell lines were also grown in MCDB 170. hTERT-transduced 184 HMEC and 184A1 were generated as described (Stampfer et al., 2001).

Retrovirus production and infection

Retroviral constructs containing EGFPΔRaf-1:ER, an estrogen-inducible form of Raf-1 fused to enhanced green fluorescent protein (EGFP) in the pBabePuro3 (pBP3) vector (Woods et al., 1997; Zhu et al., 1998), as well as the control construct encoding the estrogen receptor hormone binding domain only (ER), were provided by M. McMahon (UCSF Cancer Center, San Francisco, CA). Constructs containing HPV16-E6 (D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA), HPV16-E7 (V. Band, Tufts University, Boston, MA), SV40 large T antigen (J. Campisi, LBNL), adenovirus E1A (S. Frisch, La Jolla Cancer Res. Foundation, La Jolla, CA), and c-myc (B. Amati, DNAX Research Institute, Palo Alto, CA) were obtained in LXSN or LNSX retroviral vectors. Amphotropic viruses were prepared by transient co-transfection of HEK293 cells with retroviral and packaging plasmids (Finer et al., 1994). Parallel HMEC cultures were infected with experimental or control retroviruses and selected in 2 μg/ml puromycin or 300 μg/ml G418 for 7-10 days.

Growth assays and flow cytometric analysis

Cell counts were performed by seeding cells into triplicate 35-mm dishes at 2 x 10^4 cells per dish. 24 hours after seeding, a day 0 sample was trypsinized and counted in a Coulter counter, and treatment of the remaining dishes with ethanol or 4-HT was initiated. After 4 or 6 days, cells were counted and relative growth calculated after subtracting the number of cells present at day 0. Labeling index was determined by incubating cells with [^{3}H]-thymidine (0.7 μCi/ml; Amersham) for 24 hours prior to fixation, then visualizing and quantitating labeled cells as described (Stampfer et al., 1993).

Colony-forming efficiency was determined by seeding 50-2000 cells, which had been previously exposed to ethanol or 100 nM 4-HT for 6 days, into 100-mm dishes without ethanol or 4-HT. Cells were allowed to grow for 2 weeks and then stained with 0.1% crystal violet in 25% ethanol. Colonies composed of more than 50 cells were counted.

Anchorage-independent growth was assayed by seeding $3x10^5$cells in 1.5% methylcellulose in MCDB 170 on dishes coated with Polyhema (Sigma). Plates were fed weekly, and colonies of diameter 80-150 μm or >150 μm were counted using a calibrated ocular grid after three weeks as described (Stampfer & Bartley, 1985).

EGF-independent growth of 184A1- and 184B5-Raf:ER was assessed by pre-treating cells for one passage with either ethanol or 100 nM 4-HT, then seeding them as for cell counts. Half of the dishes received complete MCDB 170, while the other half received medium minus EGF, plus 5 μg/ml of anti-EGF receptor mAb225 (Stampfer et al., 1993). After 6 days, cells were counted.

Flow cytometric analysis was performed on cells treated for 6 or 12 days with either ethanol vehicle alone or 100 nM 4-HT. Cells were trypsinized, collected into 70% ethanol, and stained with propidium iodide as described (Krtolica & Ludlow, 1996). DNA content was analyzed on a Beckman-Coulter EPICS XL flow cytometer. Cell cycle analysis was performed using the MCycle software (Phoenix Flow Systems). 184A1-Raf:ER cells at passage 74 were sorted on the basis of their EGFP expression using a Beckman-Coulter EPICS Elite cell sorter. The cells expressing the highest levels of EGFP fluorescence (~3% of the starting population) were isolated and used in further experiments.

SA-β-gal, viability, and TUNEL staining

Cells were stained for SA-β-gal as described previously (Dimri et al., 1995). Briefly, cells were rinsed with PBS, fixed for 5 minutes at room temperature with 3% formaldehyde, and stained overnight at 37°C with a solution consisting of 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide/ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$.

To determine cell viability, MTT assays were performed as previously described (Alley et al., 1988), and trypan blue staining was performed as follows. One ml of a 1:1 solution of 0.4% trypan blue/PBS was added directly to 35-mm dishes of cells, incubated at 37°C for 5-10 minutes, then aspirated off the cells. Random microscopic fields of cells were counted, and the percentage of clear (viable) vs. blue (non-viable) cells was determined.

TdT-mediated dUTP nick end-labeling (TUNEL) staining in control and Raf:ER-arrested cells was assayed using the DeadEnd Colorimetric Apoptosis detection kit (Promega).

Western blot analysis

Total cell lysates were made by lysing cells into 2% SDS, 10% glycerol, and 0.063 M Tris-HCl (pH 6.8) containing protease inhibitors (20 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A; Boehringer Mannheim, Indianapolis, IN). Lysates were heat denatured at 95°C for 10 minutes, passed through a 26G needle to shear DNA, and centrifuged at 10,000 g for 30 minutes to remove insoluble material. Protein concentrations were determined using the BCA assay (Pierce). 50 μg of each sample was resolved on 4-15% polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell). Even loading of protein samples was verified by staining with Ponceau S. After blocking with 5% nonfat dry milk, 0.05% Tween-20 in Tris-buffered saline, blots were incubated with anti-p16 (Ab-1, NeoMarkers), anti-p21 (Pharmingen), anti-p53 (Ab-6, Calbiochem), anti-p27 (C-19, Santa Cruz), anti-p57 (C-20, Santa Cruz), or anti-phospho-MEK (Cell Signaling Technology) antibodies. Antibody-antigen complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and the SuperSignal West Pico chemiluminescent substrate (Pierce).

Northern blot analysis

A Northern blot containing 10-μg samples of total RNA purified from cell cultures using the RNeasy Mini kit (Qiagen) was hybridized to a random-primed, [^{32}P]-labeled, 1.8-kb EcoRI/XhoI fragment of p15 cDNA (G. Hannon, Cold Spring Harbor Lab., Cold Spring Harbor, NY) or cyclin D1 cDNA (S. Reed, Scripps Research Institute, La Jolla, CA). Even loading of RNA samples was verified by staining with ethidium bromide. Hybridization of the probe to the various samples was quantitated using a Storm phosphoimager and Imagequant software (Molecular Dynamics).

Reverse-transcriptase-polymerase chain reaction (RT-PCR) amplification

Total RNA purified from cell cultures as described above was analyzed by RT-PCR as previously described (Gardie et al., 1998). Amplification of p14 cDNA was performed with 2.5 mmol/L $MgCl_2$ and 5% formamide. An initial denaturation step of 3 minutes at 94°C, followed by 26 cycles consisting of 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C, with a final extension of 10 minutes at 72°C, were performed in a PCR-Express thermal cycler (Hybaid Limited, Ashford, UK). p14 cDNA was amplified using primers OL27: 5'-ATGGTGCGCAGGTTCTTGGT-3' and OL28: 5'-TGCACGGGTCGGGTGAGAGT-3'.

ACKNOWLEDGMENTS

We thank Martin McMahon for providing the EGFPΔRaf-1:ER and hbER retroviral vectors, as well as helpful discussions. We are grateful to Denise Galloway, Vimla Band, Judy Campisi, and Steve Frisch for providing viral oncogene constructs, and to Bruno Amati for the c-myc construct. We also thank Ana Krtolica and Hector Nolla for assistance with flow cytometry. This work was supported by the California Breast Cancer Research Program grant #4JB-0119 (MS, CO, PY), Association pour la Recherche Contre le Cancer (BG), NIH grant CA-24844 (MS, PY), and the Office of Energy Research, Office of Health and Environmental Research, U.S. Department of Energy under Contract No. DE-AC03-76SF00098 (MS, PY).

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Table 1: Oncogenic Raf confers anchorage-independent growth on fully immortal 184A1.

		# colonies			
Treatment	**# cells seeded**	**80-150 µm**	**≥150 µm**	**Total colonies**	**% AIG**
EtOH	**$1x10^5$**	**0**	**0**	**0**	**0**
100 nM 4-HT	**$3x10^5$**	**8**	**2**	**10**	**0.003**

184A1-Raf:ER at passage 75 were seeded into methylcellulose ± 100 nM 4-HT and refed weekly. After 3 weeks, colony numbers and size ranges were determined.

FIGURE LEGENDS

Figure 1: Activated Raf:ER inhibits the growth of post-selection 48R HMEC. Strain 48R was transduced at passage 9 and assayed at passage 15. The cells were transduced with hbER (control; A,B) or Raf:ER (C,D) retroviral vectors and treated with ethanol (A,C) or 100 nM 4-HT (B,D). Scale bar, 100 μm. In E), counts of cells treated with ethanol or 10-500 nM 4-HT for 6 days, or colony-forming efficiency (CFE) of cells pretreated with ethanol or 100 nM 4-HT for 6 days at passage 16, then seeded at colony-forming density and allowed to grow for two weeks, are plotted as a percentage of control (ethanol-treated) cell counts. For CFE, colonies of >50 cells were counted.

Figure 2: Labeling index and cell cycle analysis of post-selection 48R-Raf:ER and controls. A) 48R transduced with Raf:ER or hbER (control) retroviral vectors were treated at passage 15 with ethanol or 10 nM, 100 nM, or 500 nM 4-HT for 6 days and labeled with ^{3}H-thymidine for the final 24 hours of treatment. Labeling index was then determined, with results plotted as a percentage of control (ethanol-treated) cells. B) 48R transduced with Raf:ER or control retroviral vectors were treated at passage 17 with ethanol or 100 nM 4-HT for 6 days, then stained with propidium iodide andanalyzed by flow cytometry to determine cell cycle distribution. An additional Raf:ER-transduced sample was induced for 12 days prior to analysis. Data are presented as percentage of cells in each phase of the cell cycle.

Figure 3: Expression of cell cycle regulators in post-selection 48R-hbER and -Raf:ER. Cells at passage 15 were treated with ethanol or 100 nM 4-HT for 48 hours, then expression of the indicated cell cycle regulators was determined by Western blot (p16, p21, p53, p27, p57, MEK phosphorylation), Northern blot (cyclin D1, p15), or quantitative RT-PCR (p14).

Figure 4: Response of pre-selection 48R HMEC and 48R fibroblasts (Fb) to Raf:ER. Pre-selection 48R HMEC (A) or 48R Fb (B) were transduced with Raf:ER retroviruses at passage 2 or 6, respectively, then treated with ethanol or 100 nM 4-HT for 6 days. C) Cell counts and SA-β-gal staining of 48R HMEC or 48R Fb were determined after 6 days' treatment with ethanol or 100 nM 4-HT. D) Relative expression of the indicated cell cycle regulators was determined by Western blot after treatment with ethanol or 100 nM 4-HT for 48 hours. Scale bars, 100 μm.

Figure 5: Viral oncogenes or overexpression of c-myc does not abrogate Raf-induced growth inhibition in post-selection 48R HMEC. Cells transduced with Raf:ER plus empty vector (LXSN) or vectors encoding HPV16-E6, -E7, SV40T, adenovirus E1A, or c-myc were treated at passage 17 or 18 with ethanol or 4-HT for 6 days, then counted. All data are presented as a percentage of control (ethanol-treated) cells.

Figure 6: Fully immortal and p53(-/-) HMEC are resistant to Raf-induced growth arrest. 184A1-Raf:ER at passage 13 (pre-conversion conditionally immortal, A) or passage 71 (fully immortal, B), and 184B5-Raf:ER at passage 16 (conditionally immortal, C) or passage 48 (fully immortal, D) were treated with ethanol or 100 nM 4-HT for 6 days, then photographed, counted, or seeded at low density to determine colony-forming efficiency (CFE). E) The p53(-/-) cell line 184AA2-Raf:ER at passage 57 was treated as described, then photographed or counted. Cell counts and CFE are plotted as percent of control (ethanol-treated) cells. Scale bar, 100 μm.

Figure 7: Phosphorylation of MEK after induction with 100 nM 4-HT. 48R-Raf:ER at passage 16, and 184A1-Raf:ER at passage 15 (conditionally immortal) or 72 (fully immortal) were treated with 100 nM 4-HT for 0-48 hours and assayed for Raf:ER expression and MEK phosphorylation by Western blotting.

Figure 8: hTERT-immortalized HMEC which do not undergo conversion remain growth-inhibited by Raf:ER. A) 184-hTERT-Raf:ER at passage 27 or 29 were treated with ethanol or 100 nM 4-HT for 6 days,

then photographed, counted, or seeded at low density to determine colony-forming efficiency (CFE). Counts and CFE are plotted as percent of control (ethanol-treated) cells. In addition, conditionally immortal 184A1 transduced with hTERT at passage 12 (B; pre-conversion) or 22 (C; in-conversion) were transduced with Raf:ER and treated with ethanol or 4-HT for 6 days, then photographed and counted. Scale bar, 100 μm.

Figure 9: Oncogenic Raf confers EGF-independent growth on fully immortal 184A1. 184A1-Raf:ER at passage 74 were grown for one additional passage in the presence or absence of 100 nM 4-HT. Cells were then sub-cultured and allowed to grow for 24 hours, after which they were refed regular (+EGF) medium or medium minus EGF plus MAb 225. After 6 days, cells were counted.